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The Putative Neuraminyllactose-Binding Hemagglutinin HpaA of *Helicobacter pylori* CCUG 17874 Is a Lipoprotein

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The ability of certain strains of *Helicobacter pylori* to cause sialic acid-sensitive agglutination of erythrocytes has been attributed to the HpaA protein (D. G. Evans, T. K. Karjalainen, D. J. Evans, Jr., D. Y. Graham, and C.-H. Lee, J. Bacteriol. 175:674–683, 1993), the gene for which has been cloned and sequenced. On the basis of the hydropathy plot of HpaA and the presence of a potential lipoprotein signal sequence and modification site, and because of the similarities of these features with those of the cell envelope lipoprotein Lpp20 of *H. pylori*, we examined the possibility that HpaA was also a lipoprotein. Posttranslational processing of the HpaA protein expressed by the cloned gene was sensitive to globomycin, an inhibitor of the lipoprotein-specific signal peptidase II. Antibodies raised to the putative sialic acid-binding region of HpaA failed to bind to the surface of *H. pylori* cells in immunoelectron microscopy but instead were observed to have labeled the cytoplasm when thin sections were examined. This antibody recognized a 29,000-M_r protein in Western blots (immunoblots) of cell extracts of *H. pylori* and *Escherichia coli* cells expressing the cloned *hpaA* gene. Determination of the sequence of *hpaA* from strain CCUG 17874 indicated significant differences from that determined by Evans and coworkers in the above-mentioned study, including extension of the gene into the open reading frame 3 downstream of *hpaA* to produce a protein with an M_r of 26,414. Localization of HpaA indicated that it was predominantly located in the cytoplasmic fraction of the cell in both *E. coli* and *H. pylori*. HpaA was not observed in the sarkosyl-insoluble outer membrane fraction. An isogenic mutant generated by insertional inactivation of *hpaA* was unaffected in its ability to bind four different human cell lines as well as fixed sections of gastric tissue and had hemagglutination properties identical to those of the wild type. The data collectively suggest that HpaA is a nonessential lipoprotein internal to the *H. pylori* cell and that it is not involved in adhesion.

The ability of bacterial pathogens to bind specific targets, often glycolipids, on eukaryotic cell surfaces is an important aspect of the colonization process and is mediated by bacterial receptors or adhesins (30). Such interactions are also likely to be important for persistence in chronic infections. The human gastric pathogen *Helicobacter pylori* has been associated with gastritis and gastric and duodenal ulcers (22, 35, 49). There is an increasing body of evidence for a linkage between colonization with the human pathogen *H. pylori* and gastric cancer (5, 49, 52). There is clear evidence that *H. pylori* binds to gastric epithelial cells and cultured cells and exhibits tropism in its pattern of colonization of the gastric mucosal epithelium (4, 9, 20, 36, 50). Specific receptors indicated by these studies include phosphatidyl ethanolamine (36) and fucose-containing glycoproteins (7, 20). A protein with an M_r of 63,000 has been identified as the *H. pylori* binding component for phosphatidyl ethanolamine, and a unique amino-terminal amino acid sequence was also determined (37). The recently characterized affinity of *H. pylori* for fucose-containing compounds, exemplified by the Lewis B human blood group antigen, has demonstrated a physical correlation between the pattern of bacterial binding and the distribution of this antigen in fixed sections of gastric tissue (7, 20).

Early investigations of receptor recognition by *H. pylori* focused primarily on hemagglutination rather than gastric tissue binding because of the simplicity of the former assays and their potential value in identifying adhesins. Patterns of erythrocyte agglutination and sugar inhibition have consistently identified *N*-acetylneuraminyllactose (NANA), or sialic acid, as a likely receptor and as one recognized by a substantial proportion of strains (6, 15–17, 26).

The putative NANA hemagglutinin HpaA has been identified and characterized by Evans and coworkers (17, 18). HpaA was reported to have a subunit molecular weight of 20,000, to possess NANA-sensitive hemagglutination, and to have a putative filamentous structure (17). Subsequently, the sequence of a chromosomal region including the open reading frame (ORF) for HpaA and two flanking genes was determined (18). The *hpaA* gene had no significant homologs in the databases but appeared to include a hexapeptide motif shared by well-characterized bacterial sialic acid-recognizing hemagglutinins. Furthermore, lysates of *Escherichia coli* cells harboring the cloned gene bound to the NANA-containing glycoprotein fetuin, as did purified HpaA.

We observed that the published sequence for HpaA had a typical lipoprotein cleavage and modification consensus motif, had no potential membrane-spanning regions apart from the presumptive signal, and in these and other respects was similar to Lpp20, a nonessential envelope lipoprotein from *H. pylori* that we have recently characterized (32). To examine the pos-

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sibility that HpaA was a lipoprotein and to evaluate the contribution of HpaA to the hemagglutinating and adhesive properties of *H. pylori*, we cloned, sequenced, expressed, and inactivated the *hpaA* gene from a type strain of *H. pylori*. The sizes of the gene and gene product differed significantly from those of strain 8826 of Evans and coworkers (18). We present evidence suggesting that HpaA in strain CCUG 17874 is a lipoprotein localized predominantly in the inner membrane and cytoplasm. Surface exposure of HpaA could not be demonstrated immunologically. An *hpaA* mutant of *H. pylori* was unaffected in cell adhesion, hemagglutination, and glycolipid binding, opening to question the role, if any, of HpaA in these processes in vivo.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pylori* strains examined in this study were endoscopic biopsy isolates from the Culture Collection University of Göteborg, CCUG 915 (Stockholm, Sweden, isolate) and CCUG 17874. CCUG 17874 is identical to the type strain NCTC 11637, isolated by B. J. Marshall at Royal Perth Hospital, May 1982 (39). Also used were strains 5294, 5442, and 5155 (Sydney, Australia, isolates), strains 7958 and 5790 (Canadian isolates from the culture collection of T. J. Trust), and strain A5 (Swedish isolate; Astra Arcus AB, Södertälje, Sweden). A derivative of strain A5, designated A5-nu, was obtained by serial passage of strain A5 through a nude mouse (28). *Helicobacter mustelae* 180, 181, and 4298 (42), *Helicobacter felis* strain Hf1, *Campylobacter rectus* strains 33238 and 314 (13), *Campylobacter coli* strain VC167B (25), and *E. coli* strain JM109 were also examined. Stock cultures were maintained at −70°C in 15% (vol/vol) glycerol-Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Cultures of *Helicobacter* spp. were grown at 37°C on chocolate-blood agar (CBA) plates in an atmosphere containing 10% (vol/vol) carbon dioxide for 48 h. For growth of cells for the interleukin stimulation assay, cultures were grown in brucella broth supplemented with 2% (vol/vol) fetal calf serum (containing 20 μg of kanamycin per ml for cultivation of the *hpaA* mutant) and incubated at 37°C for 18 h in a microaerobic atmosphere with shaking at 150 rpm. *C. rectus* was grown on CBA under anaerobic conditions at 37°C. *C. coli* was grown on Mueller-Hinton agar at 37°C in an atmosphere containing 10% (vol/vol) carbon dioxide. *E. coli* was grown at 37°C in Luria broth. When the addition of globomycin was required, it was added from a stock solution of 10 mg/ml in ethanol.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (34) in a minislab apparatus. Protein was stained with Coomassie blue R-250. Western blotting (immunoblotting) was performed as previously described (45).

Electron microscopy. For negative staining, a grid covered with a Formvar film was floated on a 50-ml drop of bacterial cells in Tris-buffered saline (TBS) for 5 min. The grid was stained by floating on a drop of 1% (wt/vol) ammonium molybdate, pH 7.5, and was examined in a JEOL JEM-1200EX electron microscope operated at an accelerating voltage of 75 kV.

For immunoelectron microscopy, the grid was floated on the sample, removed, and floated on a drop of TBS containing 1% (wt/vol) bovine serum albumin (BSA) for approximately 30 min. The grid was then incubated on a drop of TBS containing the diluted antibody for 1 h. After incubation, the grid was removed and nonspecifically bound immunoglobulin was removed by floating the grid on three drops of TBS. The grid was then floated on a drop of TBS containing a 1:50 (vol/vol) dilution of 15-nm-diameter colloidal gold particles coated with protein A (Amersham Canada, Oakville, Ontario, Canada). After incubation for 30 min, the nonspecifically bound colloidal gold particles were removed by floating the grid on three drops of TBS. The grids were negatively stained and examined as above.

Samples of *H. pylori* CCUG 17874 were prepared for sectioning as follows. Bacteria were grown on CBA for 2 days as described above. Bacteria were fixed for 1 h by suspension in 2.0% paraformaldehyde–0.7% glutaraldehyde–0.1 M cacodylate buffer, pH 6.9. Cells were then washed twice in 0.1 M cacodylate buffer (pH 6.9) and suspended in cacodylate buffer containing 50 mM ammonium chloride for 30 min. Bacteria were collected by centrifugation (13,000 × *g* for 10 min), and the pellet was enrobed in 2% Noble agar and bloc stained with 2% uranyl acetate. Sample blocks of agar were infiltrated overnight in LR white resin at room temperature. The resin was changed and hardened by incubation at 60°C for 1 h. Thin sections were then cut with a Reichert OmU2 microtome, stained for 1 min with 2% uranyl acetate and then 0.2% lead citrate, and examined in a Hitachi H-7000 transmission electron microscope.

For immunogold labeling, the method employed was that described by Noonan and Trust (41), employing the antibody CR49 at a 1/50 dilution. Samples were stained and examined as described above.

Immunofluorescence. Immunofluorescent bacterial cell surface labeling was assessed with affinity-purified antipeptide immunoglobulin G (IgG) (1:200 dilution) as described by Doig and Trust (13).

Cellular localization of HpaA. Cellular localization studies were performed with affinity-purified antipeptide IgG (1:1,000 dilution) as previously described (11, 13).

Adhesion assay. For use in a cultured-cell adhesion assay, bacterial cells were grown for 3 days on CBA as described above. Cells were harvested into Dulbecco's modified Eagle medium (DMEM; Gibco, Gaithersburg, Md.) and washed once with DMEM (12,000 × *g*, 15 min, 4°C). Bacteria were resuspended in DMEM, and the density of the suspensions measured at 650 nm was adjusted to 0.2. Tissue culture cell lines were grown as described previously (10). For binding assays, cell lines were split into 24-well tissue culture plates covered with coverslips. Tissue culture cells were then grown for 2 days. Prior to use in the assay, tissue culture medium was replaced with DMEM for 1 h. Bacteria were then added to the tissue culture wells and incubated at 37°C in a 10% (vol/vol) CO_2 environment for 30 min. The wells were then washed four times with TBS by aspiration, and the cells were fixed with 100% methanol. Coverslips were then removed from the wells and mounted on microscope slides, and adherent bacteria were counted by phase microscopy. The number of bacteria bound per cell was counted in five fields, and binding assays were performed in duplicate.

Production of antisera. The antiserum prepared against whole bacterial cells of *H. pylori* CCUG 17874 has been previously described (32). For production of antiserum against synthetic peptide, a New Zealand White rabbit was injected with 100 μg of KLH (keyhole limpet hemocyanin)-conjugated peptide in Freund's complete adjuvant. After receiving two booster injections of 100 μg of antigen in Freund's incomplete adjuvant, the rabbit was sacrificed and serum was collected by cardiac puncture. The degree of peptide-specific antibody was assessed by enzyme-linked immunosorbent assay (ELISA), comparing the reactivities of the serum to both BSA- and KLH-conjugated peptides.

Specific antipeptide antibody was purified by affinity chromatography. An affinity column was made by coupling the N-terminal Cys of the peptide to a 2-fluoro-1-methylpyridinium-activated AvidGel matrix as described in the instruction manual (BioProbe International, Inc., Tustin, Calif.). Protein G-purified IgG was isolated from the immune serum and passed through the affinity column equilibrated with TBS. After the column was washed with 5 bed volumes of TBS, peptide-specific IgG was eluted with 0.2 M glycine, pH 2.0. After reequilibration of the column with TBS, further peptide-specific IgG was eluted with 10 mM triethylamine, pH 11.2. Fractions containing IgG specific for the peptide (as determined by ELISA with BSA- and KLH-conjugated peptides as the antigens) were pooled and dialyzed against TBS. This antibody preparation reacted with BSA- and KLH-conjugated peptides but failed to recognize unconjugated BSA and KLH in Western immunoblots. This antibody (termed CR49) was stored at −20°C until use.

Synthesis of synthetic peptide. The peptide corresponding to the putative sialic acid-binding domain of HpaA with an added N-terminal cysteine (C-L-R-P-D-P-K-R-T-I-Q-K-K) was synthesized by the Merrifield solid-phase methodology on an Applied Biosystems model 430A automated peptide synthesizer. Peptides were conjugated to Imject (maleimide)-activated KLH or BSA via the N-terminal Cys of the peptide by the method described in the instruction manual (Pierce Chemical Co., Rockford, Ill.).

PCR and molecular cloning. PCRs were performed in a standard reaction buffer (10 mM Tris-Cl, pH 8.3, 50 mM KCl) including 2.5 mM $MgCl_2$, 200 μM all four dNTPs, 200 nM each primer, and 0.5 U of *Taq* polymerase (AmpliTaq; Perkin-Elmer Cetus Corp., Norwalk, Conn.) with a Perkin-Elmer model 9600 thermal cycler. After a preamplification denaturation step of 95°C, 2 min, reactions were incubated for 30 cycles of 94°C, 1 min; T_a, 1 min; 72°C, 1 min (where T_a, the annealing temperature for the reaction, was 2 to 4°C below the lower melting temperature of the two primers). The primers used to amplify the *hpaA* gene were 5′-GGGGATCCGAGTGGAGGCAAAAGCGGG-3′ and 5′-GGGAATTCGCATAGGCTTGAATGGGTGG-3′. These primers amplify residues 418 to 1180 of the *hpaA* gene (accession no., X61754 [18]). The primers used in the inactivation of *hpaA* were HPADEL-1, 5′-CCATCGTAGATATCGGGCTGCATCCCACTAATAAAGC-3′; HPADEL-2, 5′-GCAGCCCGATATCTACGATGGATTTGAGCGAGTTGGAC-3′; DYNA, 5′-AAAGGGGGATGTGCTGCAAGGCG-3′; and DYNB, 5′-GCTTCCGGCTCGTATGTTGTGTG-3′ (27).

DNA was isolated from *H. pylori* and *H. mustelae* by a published modification (44) of the guanidine lysis protocol of Pitcher and coworkers (46). Standard procedures were employed for plasmid cloning experiments with *E. coli* (48). The plasmids pUC18 (55) and pK18 (47) were also employed. Sequence reactions were performed on double-stranded templates with *Taq* polymerase (Perkin-Elmer Cetus), dye-labeled terminators, and a Perkin-Elmer Cetus model 480 thermal cycler for analysis with the model 373A automated sequencer (ABI, Foster City, Calif.). Sequence data were collated and assembled with the Geneworks Package (Intelligenetics, El Camino, Calif.) and analyzed with MacVector software (IBI, New Haven, Conn.). The plasmid pILL600 used as the source of kanamycin resistance has been described previously (33). *E. coli* strains were tested for expression of cloned antigen by resuspension of a cell pellet containing approximately 5×10^7 stationary-phase cells in 30 μl of solubilization buffer. The sample was boiled for 5 min prior to electrophoresis.

Electroporation of *H. pylori*. Bacterial cells were inoculated from CBA plates into Hams F12 cell culture medium (Gibco) supplemented with 10% (vol/vol) fetal calf serum, amphotericin B (4 mg/ml), vancomycin (10 mg/ml), and trimethoprim (5 mg/ml). Cultures were grown in 100 ml of medium in 250-ml flasks

in microaerophilic jars, at 37°C with shaking at 100 rpm. After 24 to 48 h, when the cell density had reached 10^8 CFU/ml as measured by viable counting, cells were collected by centrifugation, washed twice in 15% (vol/vol) glycerol–9% (wt/vol) sucrose, and finally resuspended in a 1/5,000 volume of the original culture in glycerol-sucrose. Electrocompetent cells were stored at −70°C.

Electrocompetent cells were thawed on ice, mixed with 1 to 2 μg of DNA, transferred to a 0.1-cm-diameter cuvette, and subjected to one pulse (25 μF, 200 Ω, and 1.5 kV, giving a time constant of 4.5 to 4.8 ms) in a Bio-Rad electroporator. Cells were immediately resuspended in 10 ml of medium and incubated for 16 h at 37°C with shaking in a microaerophilic environment. Bacterial cells were collected by centrifugation and plated on CBA plates containing 20 μg of kanamycin per ml.

Southern hybridization. Southern hybridization was performed at 68 or 38°C (when high or moderate stringency, respectively, was required) in 6× SSC (48) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) incorporating 10× Denhardt's solution, 0.25 mg of yeast tRNA per ml, 1% (wt/vol) SDS, and 2 mM EDTA. Blots were washed twice in 2× SSC–0.5% (wt/vol) SDS at 68°C for 15 min and twice in 1× SSC–0.1% (wt/vol) SDS at room temperature for 15 min (high stringency). When conditions of moderate stringency were required, the filters were washed twice in 2× SSC–0.5% (wt/vol) SDS at 38°C for 15 min. The probes for the 16 S and 23 S ribosomal genes of *H. pylori* were obtained by PCR with primers 16S-1, 5′-TCCTGGCTCAGAGTGAACGCT-3′; 16S-2, 5′-GGAC TACCAGGGTATCTAATC-3′; 23S-1, 5′-GTCGGGTAAGTTCCGACCT-3′; and 23S-2, 5′-GGCGAACAGCCATACCCTT-3′; as described by Taylor and coworkers (53).

Hemagglutination assay. Cells were cultured for 48 h on CBA plates, harvested with a cotton swab, and resuspended in 10 mM sodium phosphate–0.15 M NaCl, pH 7.2 (PBS) to a final optical density at 600 nm of 1.8. Bacterial suspensions in PBS (20 μl) were serially diluted twofold in round-bottomed 96-well trays prior to the addition of 20 μl of a 2% (vol/vol) suspension of guinea pig erythrocytes. The tray was agitated gently and incubated for 1 h at room temperature before visual inspection for hemagglutination. For testing the inhibitory effect of NANA (2-3 isomer; Sigma Chemical Co., St. Louis, Mo.), this compound was included in the wells at a final concentration of 330 ng/μl.

In situ binding assay. Cells of *H. pylori* were tested for the ability to bind to fixed slices of human gastric tissue as described previously (20).

Lipid extraction and binding assays. Lipids were extracted from the mucosa of a pig stomach antrum according to the method reported by Karlsson (31). A standard thin-layer chromatography separation was performed (37). In brief, lipids were separated on silica gel-coated plates (Whatman Ltd., Maidstone, England) with chloroform-methanol-water (65:35:8, vol/vol/vol). The plates were blocked with 1% (wt/vol) polyvinylpyrrolidone–1% (wt/vol) gelatin in PBS at 37°C for 2 h. The plates were then incubated with bacterial suspensions (approximately 5×10^7 cells/ml) in PBS containing 0.25% (wt/vol) polyvinylpyrrolidone–0.25% (wt/vol) gelatin at 37°C for 90 min. After being washed with PBS, the plates were incubated with rabbit antiserum to whole cells of *H. pylori* CCUG 17874 diluted in PBS–0.25% (wt/vol) polyvinylpyrrolidone–0.25% (wt/vol) gelatin at 37°C for 60 min and then were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Caltag Laboratories Inc., South San Francisco, Calif.). The plates were washed and developed in the substrate solution as described for Western blotting.

Binding to lipids immobilized in ELISA plates was also performed. Wells were coated overnight at 4°C with the extracted lipids in methanol and then were washed with PBS and blocked with 2% (wt/vol) BSA in PBS at 37°C for 90 min. After the wells were washed three times, bacterial suspensions (approximately 5×10^7 cells/ml) in PBS or 2% (wt/vol) BSA (control) were added and the plates were incubated at 37°C for 90 min. After the wells were washed three times, the plates were incubated with rabbit antiserum to whole cells of *H. pylori* CCUG 17874 followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (Caltag Laboratories Inc.). The plates were developed with disodium *p*-nitrophenyl phosphate (Sigma) solution and read at 405 nm in an ELISA reader as described previously (13).

Nucleotide sequence accession number. The sequence of the *hpA* gene of *H. pylori* CCUG 17874 has been submitted to GenBank and is listed under accession number U35455.

RESULTS

Production of antiserum specific for HpaA. Evans and coworkers have previously described a dodecapeptide, containing the putative receptor-binding motif of HpaA, which they used for the production of a specific antibody (18). Accordingly, this synthetic peptide was coupled to KLH and used for immunization. Dodecapeptide-specific antibody was purified by affinity purification. The resulting antiserum had an ELISA titer of 8,000 for BSA-conjugated peptide and reacted by Western immunoblot with a single 29,000-M_r protein in *H. pylori* cell lysates. This corresponded to a major antigen also recognized by antiserum raised against whole cells of *H. pylori* CCUG 17874 (Fig. 1).

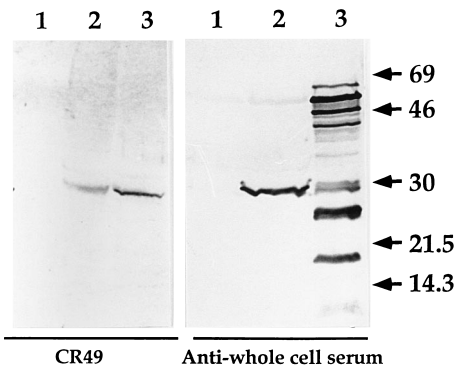

FIG. 1. Production of antiserum specific for HpaA. The samples indicated were separated by SDS-PAGE and probed with affinity-purified antibody raised against a dodecapeptide including the putative receptor motif of HpaA (CR49) or antiserum against whole cells of *H. pylori* CCUG 17874. Whole-cell lysates were loaded as follows: lanes 1, *E. coli* DH5α; lanes 2, *E. coli* DH5α/pHPA5; lanes 3, CCUG 17874. The relative positions of the molecular weight markers (in thousands) are listed on the right.

Molecular cloning and expression of the *hpaA* gene of *H. pylori*. Using a pair of primers based upon the published sequence of *hpaA*, we amplified a 0.7-kb fragment from the chromosome of *H. pylori* CCUG 17874. This fragment was purified and cloned into the plasmid vector pBluescript II KS(−) to generate the recombinant plasmid pHPA1. Limited DNA sequence analysis from each end of the cloned fragment confirmed significant homology with the published sequence of *hpaA* (43).

To directly clone the full genomic region containing *hpaA*, the fragment originally cloned in pHPA1 was labeled and used as a probe in Southern hybridization against DNA of *H. pylori* CCUG 17874 digested with various enzymes. Fragments in a size range of 4 kb, corresponding to the size of a hybridizing band generated by *Hin*dIII digestion (43), were excised and cloned into pUC18. Transformants were screened by colony hybridization with the probe from pHPA1. One reacting plasmid, pHPA5, was thus identified. Lysates of *E. coli* cells harboring pHPA5 produced an immunoreactive band with an apparent molecular weight of 29,000 when reacted with both the antiserum raised against whole bacterial cells of *H. pylori* CCUG 17874 and the dodecapeptide-specific antiserum (Fig. 1).

Sequence determination of the *hpaA* gene of CCUG 17874. The DNA sequence of the *hpaA* gene in plasmid pHPA5 was determined with custom DNA primers. For most of the coding sequence of CCUG 17874 corresponding to the previously reported *hpaA* from strain 8826 (18), divergence was minor and resulted in either no change or conservative changes in the amino acid sequence of the gene product. However, a significant deviation between the sequences of *hpaA* of CCUG 17874 and strain 8826 in the region encoding the carboxy terminus of HpaA was noted. Significant differences between these sequences, including extra nucleic acid bases at positions 540 and 602, resulted in an extension of the *hpaA* ORF from *H. pylori* CCUG 17874 to link it in frame to an ORF just downstream (labeled ORF 3 by Evans and coworkers [18]) (Fig. 2). The primary translation product of this CCUG 17874 ORF was a protein with an M_r of 29,289.

***H. pylori* HpaA is a lipoprotein.** The HpaA protein deduced from the DNA sequence showed a number of striking similarities to a major *H. pylori* lipoprotein recently characterized by

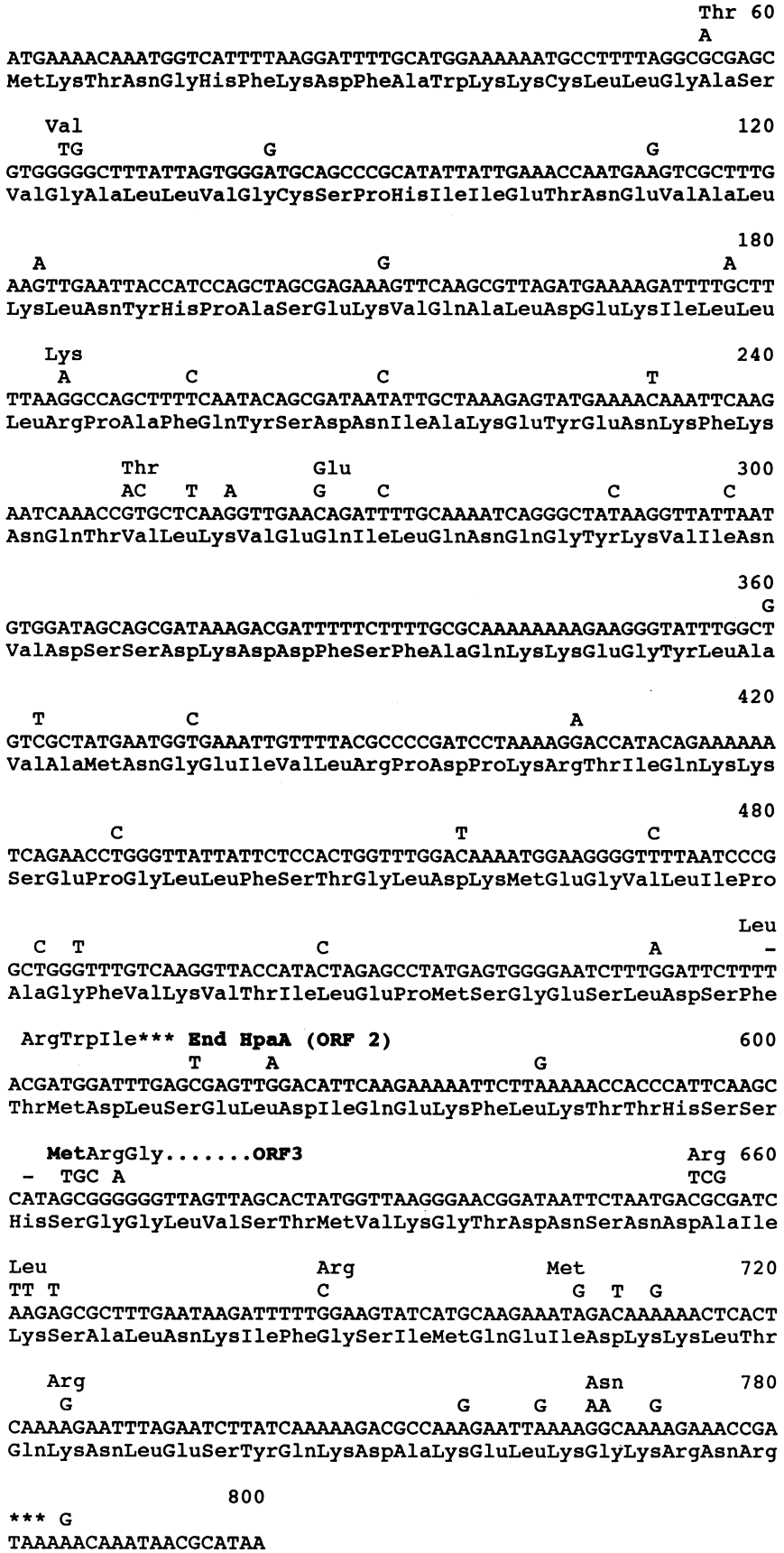

FIG. 2. DNA sequence of the *hpaA* gene of *H. pylori* CCUG 17874 and the deduced amino acid sequence of its gene product. Divergent nucleotides and their resulting change(s) in the amino acid residue(s) for the *hpaA* gene of strain 8826 are given above the CCUG 17874 DNA sequence, as are the end of *hpaA* and the beginning of ORF 3 in the sequence for that strain.

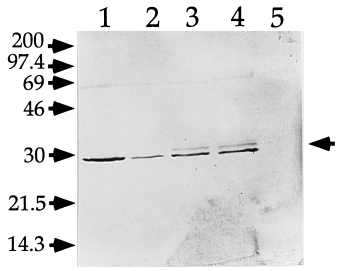

FIG. 3. Effect of globomycin on *H. pylori* HpaA processing in *E. coli*. Results of Western immunoblotting of SDS–12.5% polyacrylamide gels of whole-cell lysates of DH5α/pHPA5 cultured in the absence (lane 1) or presence of globomycin at 10 μg/ml (lane 2), 12.5 μg/ml (lane 3), and 15 μg/ml (lane 4) and DH5α (lane 5) as a control and reacted with a 1:1,000 dilution of rabbit polyclonal antibody against a dodecapeptide of HpaA are shown. The relative positions of molecular weight markers (in thousands) are listed on the left. The position of unprocessed HpaA is marked by an arrow on the right.

Kostrzynska et al., Lpp20 (32). The overall hydropathy plots are similar, and as candidate membrane-associated proteins, neither protein has a predicted membrane-spanning helix (43). For both proteins, the most strongly hydrophobic domain is at the amino terminus. This domain in HpaA is immediately followed by an LVGCS motif, a perfect lipoprotein signal cleavage and modification sequence (54), similar to that of Lpp20 (32). To test the possibility that HpaA is a lipoprotein, *E. coli* cells harboring the cloned gene were grown in liquid broth containing increasing amounts of globomycin, a cyclic peptide antibiotic which inhibits bacterial signal peptidase II. As the concentration of the antibiotic increased, there was a shift in the apparent molecular weight to a larger form, that retaining the signal peptide, as shown by SDS-PAGE (Fig. 3). Repeated attempts to demonstrate an effect of globomycin in *H. pylori* were unsuccessful, which is in keeping with recent findings of Kostrzynska et al. for another *H. pylori* lipoprotein, Lpp20 (32).

Subcellular localization of HpaA in *H. pylori*. The subcellular localization of proteins in *H. pylori* has proved difficult, presumably because of the apparent ease with which proteins that are invariably cytoplasmic in other species may leak out of the *Helicobacter* cell and become associated with the surface (3, 11, 51). To tackle this problem, we have recently established a fractionation procedure which correlates the presence of seven separate protein antigens and lipopolysaccharide in the outer membrane fraction with the reactivities of respective monoclonal antibodies with the cell surface (13). The envelope is fractionated into inner and outer membranes by sarkosyl solubilization. When this fractionation strategy was applied to *H. pylori* CCUG 17874 and the resulting fractions were tested by Western blotting with antibody against the dodecapeptide, the HpaA protein was detected primarily in the cytoplasmic fraction and to a lesser extent in the sarkosyl-solubilized inner membrane (Fig. 4). No HpaA was detected in the outer membrane fraction.

Evans and coworkers showed the presence of HpaA on the surface of unstained, glutaraldehyde-fixed *H. pylori* cells by immunoelectron microscopy, with antiserum against the synthetic dodecapeptide (18). Using an antiserum to the synthetic dodecapeptide sequence, prepared as described above, we were unable to gold label HpaA on the surface of *H. pylori* cells from strains CCUG 17874, CCUG 915, and 5155 (12). Glutaraldehyde fixation prior to labeling had no effect. Additionally, no reaction with the cell surface could be demonstrated by immunofluorescence (12). This strongly suggested that HpaA was not normally surface exposed. Immunogold labeling of thin sections of cells of strain CCUG 17874 with the antiserum specific for the HpaA dodecapeptide revealed the presence of the protein within the cytoplasm (Fig. 5) and occasionally within or associated with the inner membrane. No surface labeling was noted.

Conservation and species specificity of HpaA. To examine the distribution of HpaA production in *Helicobacter* spp. and other spiral organisms, a panel of 12 strains comprising *Helicobacter* species and various campylobacters was tested for production of the protein by Western immunoblotting. This analysis (Fig. 6) showed that a 26- to 29-kDa HpaA was produced by all *H. pylori* strains tested but not by *H. mustelae* or any of the other species tested. Since using the antibody raised against the synthetic peptide obviously allowed for missing HpaA-related proteins which had diverged in this region of the molecule, the immunoblotting analysis was repeated with the polyclonal antiserum raised against whole bacterial cells of *H. pylori* CCUG 17874. This antiserum had already been shown to react with HpaA in *H. pylori* cell lysates (Fig. 1). Western immunoblotting with the polyclonal antiserum against whole cells reproduced the distribution of HpaA production indicated in Fig. 6 (12).

Inactivation of *hpaA*. A two-step PCR-based procedure was employed to mutate the *hpaA* locus (Fig. 7). First, two PCRs were performed with plasmid pHPA5 containing the wild-type *hpaA* allele as template. Two fragments, of 1.6 and 2.1 kb, with the 3′ and 5′ ends of *hpaA* and the corresponding flanking sequences, were thus amplified (Fig. 7, top). The primers HPADEL-1 and HPADEL-2 have complementary sequences at their 5′ ends, resulting in PCR products with 21-nucleotide complementary overhangs. An *Eco*RV site is also introduced by these primers. The primers DYNA and DYNB are complementary with sequences of the pUC18 *lacZ* gene flanking the multiple cloning site.

The two PCR products in Fig. 4 were purified, mixed, and used as templates in a second PCR with primers DYNA and DYNB only. The resulting PCR product (Fig. 7, bottom) has a 450-bp deletion in *hpaA* and an additional *Eco*RV site. The ends were cleaved with *Hin*dIII, and the fragment was recloned in pUC18 to generate pHPA10. The 1.4-kb *Sma*I-released kanamycin resistance cassette of pILL600 (33) was cloned into the unique *Eco*RV site of pHPA10 to generate pHPA15.

The inactivated *hpaA* allele was transferred to *H. pylori* CCUG 915 by electroporation with plasmid pHPA15 and selection for homologous recombination of the mutated locus into the chromosome conferring kanamycin resistance. Once a

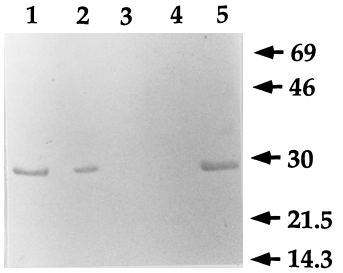

FIG. 4. Subcellular localization of HpaA in *H. pylori* CCUG 17874. Lane 1, lysate of *E. coli* DH5α harboring pHPA5; lane 2, whole-cell lysate of CCUG 17874; lane 3, sarkosyl-insoluble fraction; lane 4, sarkosyl-soluble fraction; lane 5, cytoplasmic fraction. Cellular fractions were loaded for equal protein. The blot was reacted with the polyclonal antibody against a dodecapeptide in the sequence of HpaA. The relative positions of molecular weight markers (in thousands) are listed on the right.

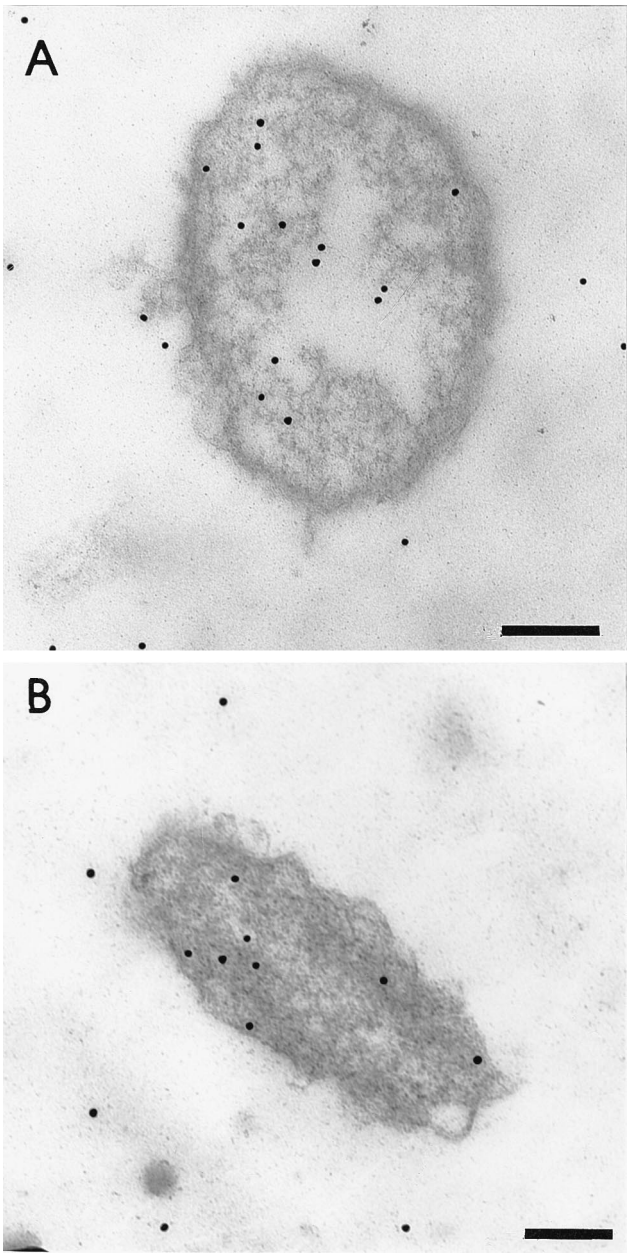

FIG. 5. Cytoplasmic localization of HpaA as demonstrated by immunoelectron microscopy of thin sections of cells of *H. pylori* CCUG 17874 incubated with antibody against a dodecapeptide from HpaA. The scale bar in each panel represents 200 nm.

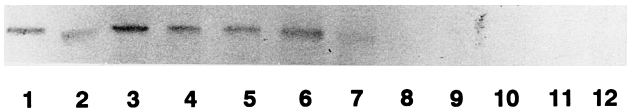

FIG. 6. Conservation and species specificity of HpaA. Fractions with equivalent protein loading of whole cell lysates of strains were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with the antibody against a dodecapeptide sequence in HpaA. Lane 1, *H. pylori* CCUG 17874; lane 2, *H. pylori* CCUG 915; lane 3, *H. pylori* 5155; lane 4, *H. pylori* 5294; lane 5, *H. pylori* 5442; lane 6, *H. pylori* 5790; lane 7, *H. pylori* 7980; lane 8, *H. pylori* CCUG 17874Δ*hpa*; lane 9, *H. mustelae* 180; lane 10, *H. felis* Hf1; lane 11, *C. coli* VC167B; lane 12, *C. rectus* 314.

putative marker exchange mutant, designated 915Δ*hpa*, had been isolated, the mutation was introduced into strains A5 and CCUG 17874 by electroporation with chromosomal DNA of the mutant 915Δ*hpa*. The mutant derivatives of these strains were characterized by Southern hybridization with probes based on *hpaA* and the kanamycin resistance marker (43). Sequences hybridizing with the Km probe were only present in the mutants, as expected. The *hpaA* probe hybridized to a 4.0-kb fragment which was increased to 5 kb in the mutants, consistent with the deletion of a 450-bp fragment and insertion of the 1.4-kb Km^r fragment. To further confirm that the mutant strains were derived from the respective parental isolates, ribotyping with specific probes for the 16 S and 23 S ribosomal genes was performed. The specific hybridization patterns obtained for the mutants supported the argument for their derivation from the parent strains (43).

Phenotypic characterization of CCUG 17874Δ*hpa*. CCUG 17874 is equivalent to NCTC 11637, a widely distributed type strain, so this mutant-parent combination was focused upon. The biological activity attributed to HpaA is neuraminyllactose-sensitive hemagglutination. When the hemagglutinating properties of CCUG 17874 and its Δ*hpa* mutant were compared, identical titers were observed (titer = 64). Furthermore, this hemagglutination activity was totally inhibited by NANA in both the parent and mutant strains (titer < 2).

Bacterial hemagglutinins frequently play a part in cell adhesion, so the Δ*hpa* mutant was quantitatively compared with the parental strain for its ability to bind to cultured eukaryotic cells. No significant differences in the number of bacteria bound per eukaryotic cell were recorded (Table 1), nor were there any apparent differences in the kinetics of bacterial binding (12). Similarly, for strains A5 and CCUG 915 and their respective mutants no significant differences in cell binding were observed (12). Furthermore, when tested for their binding to fixed slices of gastric tissue, strains A5 and A5Δ*hpa* did not show any significant differences in terms of numbers of bacteria bound or the pattern of binding (12). Additionally, the binding patterns of CCUG 17874 and its Δ*hpa* mutant for total glycolipids extracted from pig stomachs and separated by thin-layer chromatography were indistinguishable (Fig. 8).

DISCUSSION

Among the members of the genus *Helicobacter*, *H. pylori* and *H. mustelae* are distinguished by the property of intimate adhesion to gastric cells (4, 23, 24). Because of the probable importance of this adhesion for colonization and maintenance of chronic infection, considerable effort has been directed towards identification and characterization of the bacterial adhesins responsible. Using hemagglutination as a marker for adhesion, Evans and coworkers identified the HpaA protein as a potential receptor for NANA displayed by mammalian cells (17, 18). Our data suggest that at least in the CCUG 17874 type strain of *H. pylori*, loss of HpaA production does not influence adhesion in a number of model systems.

Cloning of the *hpaA* gene of CCUG 17874, identical to the widely distributed type strain NCTC 11637, and subsequent DNA sequence analysis revealed substantial differences between the *hpaA* sequence of this gene and that of strain 8826. Most importantly, the sequence from CCUG 17874 included an in-frame linkage of ORFs 2 and 3 of the *hpaA* region of strain 8826. Recently, this finding has also been reported by Jones and coworkers (29). Numerous studies involving restriction fragment length polymorphism (38) and random primer PCR analysis (1) as well as two genomic mapping studies (8,

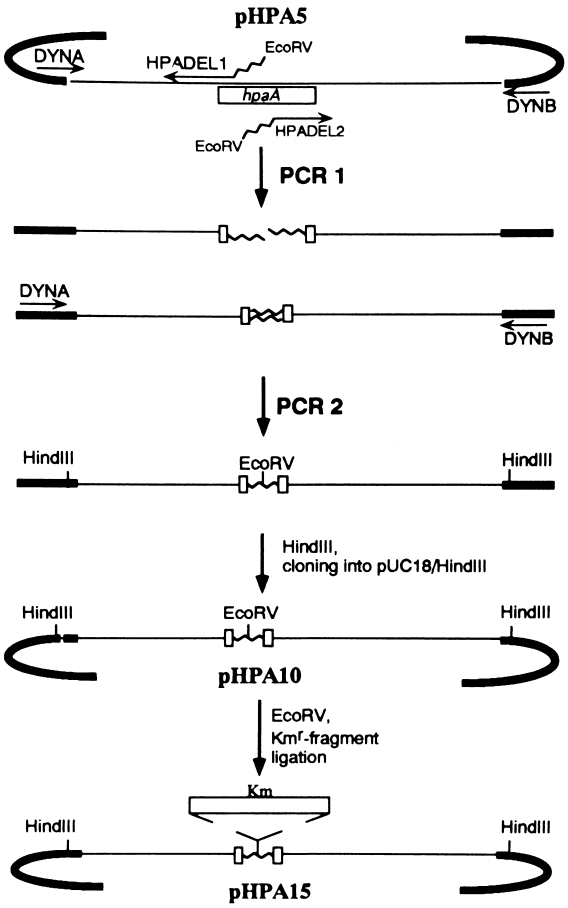

FIG. 7. Schematic representation of the strategy for deletion-insertional inactivation of *H. pylori hpaA*. Vectors are indicated by the thick black lines, and cloned *H. pylori* DNA is represented by the thin black lines. The various constructs indicated are not drawn to the same scale.

53) have all indicated that, within the species, *H. pylori* has undergone extensive chromosomal rearrangement and sequence drift. It would be unusual that this genetic diversity has proceeded to such an extent as to result in differing gene lengths for HpaA in two strains. Size heterogeneity is not unusual in surface proteins of other bacterial species, but in model systems such as staphylococcal protein A or streptococcal M proteins (21), the presence of variable numbers of repeat sequence units provides a ready explanation for the generation of size variants by homologous recombination. The reason for the size difference of HpaA in *H. pylori* strains CCUG 17874 and 8826 is unclear at present.

On the basis of the primary amino acid sequence of HpaA, we predicted and subsequently demonstrated posttranslational modification producing a lipoprotein. The deduced molecular weight of the processed gene product (26,000 exactly) was similar but not identical to that calculated by using its migration as demonstrated by SDS-PAGE (29,000). The protein produced by strain 8826 as published by Evans et al. (18) is clearly smaller since it migrates faster than the 26,500-M_r marker, but it appears larger than the 20,500-M_r product predicted by the gene sequence. This discrepancy may be due to the presumptive lipid modification, which also appears to contribute to aberrant migration of the protein from CCUG 17874.

TABLE 1. Adhesion indices for *H. pylori* strains

Cell line	Adhesion index for strain[a]	
	CCUG 17874	CCUG 17874Δ*hpa*
INT 407	28.4 ± 3.3	31.0 ± 3.3
Hep-2	52.4 ± 2.8	54.6 ± 3.5
AGS	22.2 ± 1.5	19.6 ± 3.2
KATO III	22.6 ± 1.3	25.4 ± 2.3

[a] Adhesion index reported as mean bacteria bound per cell ± standard error of the mean.

An antiserum raised against whole bacterial cells of *H. pylori* reacted strongly with HpaA expressed from the cloned gene. Since the plasmid pHPA15 might encode an antigen other than HpaA that was reactive with this antiserum and to establish that we were truly studying the same protein, a specific antiserum was raised against a synthetic dodecapeptide derived from the HpaA sequence of strain 8826. A protocol identical to that described by Evans and coworkers (18) was followed. The resulting antiserum, CR49, recognized a single band in lysates of *H. pylori* cells and *E. coli* cells harboring the cloned gene but failed to label the surface of *H. pylori* cells by immunoelectron microscopy or immunofluorescence, but the antiserum was observed to have labeled the cytoplasm when thin sections were examined, consistent with the results of cell compartment localization studies. In a further complication of the issue of the cellular localization of HpaA, Jones and coworkers (29) have reported that this protein appears to be associated with the flagellar sheath in one strain. The discrepancy between the observations made in this study and those of Evans et al. (18) and Jones et al. (29) may be due to strain differences. Alternatively, if HpaA is truly a protein internal to the cell, differences in sample preparation for microscopy may be crucial. There are numerous examples in the literature of proteins which may be visualized immunologically on the surface of *H. pylori* but which are classically internal in other genera (2, 14,

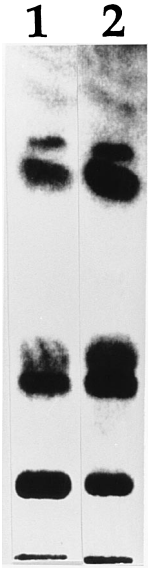

FIG. 8. Glycolipid binding assay for *H. pylori* CCUG 17874 (lane 1) and CCUG 17874Δ*hpa* (lane 2). Total glycolipids extracted from pig mucosa were separated by thin-layer chromatography, the plate was incubated with a cell suspension of the indicated bacterial strain, and bound cells were visualized by Western immunoblotting with anti-whole-cell antibody.

19, 40, 51). We suggest that these proteins and HpaA are released by *H. pylori* cells when mildly perturbed by procedures such as cell washing, in a manner similar to the release of the *H. pylori* bacteroferritin (11).

The results provided by subcellular fractionation were in strong support of the conclusions drawn above on the basis of failure to surface label the protein. Using the most discriminating protocol we could devise, we found HpaA predominantly in the cytoplasmic fraction or sarkosyl-solubilized inner membrane. Similarly, Lpp20, another strongly immunogenic lipoprotein of *H. pylori*, could not be demonstrated on the cell surface (32) but was readily released by mild extractions such as the treatment with octylglucoside employed to release HpaA. Similar properties are displayed by yet another *H. pylori* lipoprotein characterized by O'Toole et al., but one which appears to be essential for cell viability (43). These three proteins seem to be representative of a class of lipoproteins, apparently abundant within *H. pylori* cells, which are readily released by mild physical disruption. Whether or not they are important as released proteins in vivo remains to be demonstrated, though Lpp20 does not appear to provoke a strong immune response in patients (32). Like Lpp20, HpaA was produced by all strains tested and was sufficiently conserved to cross-react antigenically, suggesting evolutionary pressure to maintain and conserve the gene encoding this protein. Notwithstanding this fact, the gene could be readily mutagenized without any apparent deleterious effect, so the product is obviously not essential for survival in vitro.

The localization of HpaA to internal compartments of the cell suggested that this protein is not able to be involved in the binding of *H. pylori* to eukaryotic cells. Phenotypic analysis of the allele replacement mutant defective in HpaA production provided the strongest argument against this protein acting as an adhesin in CCUG 17874. The mutant behaved identically to the wild type with respect to hemagglutination and binding to gastric sections, separated glycolipids, or cultured cells. Admittedly, the sequence of *hpaA* from strain 8826 inferred a smaller protein which might have different localization and biological activity in that strain. Until a knockout mutant in that strain is generated, this question will remain unanswered. Given the extent to which the binding activities of HpaA have been characterized (18) and contrasting the behavior of the *hpaA* mutant, it is also possible that abolition of HpaA production is simply compensated for by the activities of other adhesins on the bacterial surface. The ability to bind phosphatidyl ethanolamine (37) and fucose-containing glycolipids (7, 20) and the complexity of hemagglutination patterns reported for the organism support the likelihood for the involvement of multiple receptors and/or adhesins.

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